

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US), CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER P66403US0
		US APPLICATION NO. (If known, see 37 CFR 1.55) 097744973
INTERNATIONAL APPLICATION NO. PCT/EP00/07843	INTERNATIONAL FILING DATE 11 August 2000	PRIORITY DATE CLAIMED 13 August 1999
TITLE OF INVENTION USE OF FIBRINOGEN MULTIMERS		
APPLICANT(S) FOR DO/EO/US Lily BAR and Israel NUR		

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

PCT Request Form

US APPLICATION NO (If known, see 37 CFR 1.5) <div style="font-size: 24pt; font-weight: bold; text-align: center;">09/744973</div>		INTERNATIONAL APPLICATION NO <div style="font-weight: bold; text-align: center;">PCT/EP00/07843</div>		ATTORNEY'S DOCKET NUMBER <div style="font-weight: bold; text-align: center;">P66403US0</div>			
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) . . \$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . \$710.00 Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) \$1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) \$100.00 Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) \$860.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS \$ 860.00		PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00			
Claims	Number Filed	Number Extra	Rate				
Total Claims	8 - 20 =	-0-	x \$18.00	\$			
Independent Claims	3 - 3 =	-0-	x \$80.00	\$			
Multiple Dependent Claim(s) (if applicable)			+ \$270.00	\$			
TOTAL OF ABOVE CALCULATIONS =				\$	990.00		
Reduction by 1/2 , Applicant qualifies for Small Entity Status.				\$			
SUBTOTAL =				\$	990.00		
Processing fee of \$130 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$			
TOTAL NATIONAL FEE =				\$	990.00		
Fee of \$40.00 for recording the enclosed assignment (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).				\$			
TOTAL FEES ENCLOSED =				\$	990.00		
				Amt. to be refunded:	\$		
				Amt. charged:	\$		
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>990.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>06-1358</u> in the amount of \$ <u>---</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. <u>06-1358</u> . A duplicate copy of this sheet is enclosed.							
SEND ALL CORRESPONDENCE TO: Jacobson, Price, Holman & Stern, PLLC 400 7th Street, N.W., Suite 600 Washington, DC 20004 202-638-6666 CUSTOMER NUMBER: 00136				By <u>William E. Player</u> William E. Player Reg. No. 31,409			

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Lily BAR et al.

Serial No.: New

Filed: February 15, 2001

For: USE OF FIBRINOGEN MULTIMERS

PRELIMINARY AMENDMENT TO LESSEN FEESAssistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS

Claim 4, line 1, delete "any of claims 1 to 3",
insert --claim 1--.

REMARKS

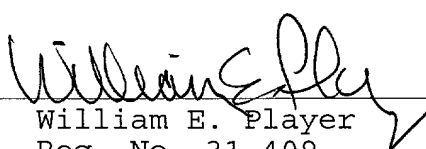
The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Early action on the merits is respectfully requested.

Respectfully submitted,

JACOBSON, PRICE, HOLMAN & STERN, PLLC

By


William E. Player
Reg. No. 31,409

400 Seventh Street, N.W.
Washington, D.C. 20004-2201
(202) 638-6666

Date: February 15, 2001
Atty. Docket: P66403US0
WEP/cmf

Use of Fibrinogen Multimers

The invention is concerned with the use of fibrinogen multimers as an ingredient of a fibrin sealant and a method of producing a fibrin sealant comprising multimers.

Fibrin sealant is a generic name covering a complex of plasma derivatives which are usually composed of proteins, which mimic those active in the last stage of the coagulation cascade, from the activation of soluble fibrinogen by thrombin to the formation of insoluble protein fibrin (Matras 1985, Sierra 1993, Spotnitz et al 1987 and Spotnitz 1995). Most of the commercial fibrin sealants are composed of two major components - thrombin supplemented with Ca^{++} ions and fibrinogen supplemented with varying amounts of factor XIII. Once both components are mixed, the thrombin splits off fibrinopeptides A and B from the respective Aa and Bb chains of fibrinogen to form fibrin monomers that are polymerized by hydrogen bonding to form a soluble fibrin clot. Factor XIII, activated by thrombin in the presence of Ca^{++} ions, catalyzes crosslinking between the fibrin molecules, increasing the mechanical strength of the clot and reducing its susceptibility to proteolytic cleavage (Gladner and Nossal 1983).

Most fibrin sealants are prepared from either plasma cryoprecipitate or from Cohn fraction I, as sources of fibrinogen. Cryoprecipitate is obtained by thawing frozen plasma at 2-4°C, resulting in a paste that is recovered by centrifugation. Cryoprecipitate contains fibrinogen, fibronectin, vonWillebrand factor and other proteins that are co-precipitated with the above proteins, such as albumin, IgG, IgM, IgA, vitamin K-dependent-clotting factors and factor XIII (FXIII). Since the use of plasma cryoprecipitate, as a source for the sealant, may result in a lower activity of factor FXIII (Radosevich et al, 1997), some of the companies add exogenous factor XIII from various sources. Products manufactured from cryoprecipitate such as TissuCol (which has recently received a free marketing authorization in the US) and BeriPlast have

been in the European market for more than a decade, however FXIII is not added to any of these products in the US. Cohn fraction I₁, or equivalent products, are prepared by ethanol precipitation of plasma by 8-10% ethanol at -30°C - -50°C at neutral pH. BioCol™ produced by Plasma Product services, Lille France is such product. The product is manufactured by processing the whole plasma. Prior to a 10% cold ethanol precipitation, the plasma is thawed at 37°C for at least 6 hours followed by recovery of the fibrinogen by centrifugation. The paste is then reconstituted in Tris/lysine buffer and subjected to a second 10% cold ethanol precipitation for an additional 10-12 hours. This allows for highly purified fibrinogen, which is then treated by solvent detergent for virus inactivation. The final product is lyophilized for prolonged storage at 4°C (Burnouf-Radosevich et al 1990).

Fibrinogen integrity and structure are of most importance for the performance of fibrin sealant. The rates of polymerization, binding to the fibrin receptor and susceptibility to fibrinolytic digestion have a critical influence on the performance in the clinical arena.

Fibrinogen, a tri-domainal (two D domains and a central E domain) disulfide bond molecule about 47 nm in length, is composed of two symmetrical half-molecules, each consisting of a set of three different polypeptide chains termed A α , B β and γ . The halves are covalently joined in the central amino-terminal E domain by five disulfide bridges. Reciprocal disulfide bridges are formed between the γ 8 and γ 9 position, thus orienting the γ chains in an anti-parallel direction (Mosesson, 1997). Removal of the fibrinopeptides A and B upon activation by thrombin induces a spontaneous polymerization of the fibrin monomer molecule via noncovalent binding.

Although fibrinogen has insufficient centers for such polymerization, nevertheless, in suitable conditions and without any enzymatic action, such as at high fibrinogen concentration, low ionic strength, or long incubation at 4°C, fibrinogen is polymerized (Rozenfeld and Vasileva 1991). Fibrinogen is liable to

undergo self-association according to some authors to form structures that are either similar to fibrin (Cohen et al 1966; Gollwitzer et al 1983; Ugarova et al 1987) or to quasi-globular particles (Becker 1987).

Rozenfeld and Vasileva (1991) found that the molecules of fibrinogen undergo a spontaneous modification of their carboxyl terminals and bind end to end into flexible polymer chains. On attaining a critical length, the single-filament polymers twist into a coil and aggregate to form branch molecules in which the segments are packed sufficiently densely to resemble strong hydrated globular particles. The formation, under the influence of factor XIIIa, of ϵ/γ -glutamyl - lysine covalent bonds produced only insignificant changes in the spatial organization of the fibrinogen aggregates. Covalent dimerization of the γ -chains restricts the structural flexibility of polymers, but linking of the α -chains provides progressive compaction of the structure with an increase in its molecular weight. Electrophoresis of reconstituted samples shows that the coil-shaped chains of fibrinogen oligomers prevent the complete enzymatic linking of the γ -chains. From the results of their work, the authors suggest that the accelerated assembly of multimolecular aggregates, seen in the presence of Factor XIIIa, may be explained by the stabilization of intermediate complexes of fibrinogen, which make the spontaneous transition from a stable native state to the active state irreversibly. However a more recent paper by Mosesson claims that fibrinogen molecules become covalently crosslinked by factor XIIIa in the same sequence as in fibrin, i.e. first the α and then the γ chain (Mega et al 1988, and Mosesson et al 1995).

It is an object of the present invention to provide a fibrin sealant having improved properties.

According to the invention fibrinogen multimers having at least 6 fibrinogen units are used as an ingredient of a fibrin sealant. Preferably, 6-15 fibrinogen units in the multimer are used. Still more preferred are 6-9 fibrinogen units. In particular 8 or 9 units are present in the multimer.

A method for manufacturing the fibrin sealant having fibrinogen multimers with at least 6 units comprises the steps of

- resuspending cryoprecipitate
- adding sucrose to yield a concentration of 60 to 70% (w/w),
- adding glycine to yield a concentration of 0.1 to 0.3 M,
- heating to 60°C for 15 - 20 hours,
- removing the glycine and the sucrose by dialysis,
- adding a protease inhibitor.

Preferably, the dialysis is conducted against a buffer comprising NaCl, glycine and CaCl_2 .

Typically, the protease inhibitor is tranexamic acid in a concentration of 8 - 12% (w/w) and/or arginine in a concentration of 1 - 3% (w/w).

Subject of the present invention is also a fibrin sealant comprising multimeric fibrinogen having at least 6 fibrinogen units.

In order to investigate the influence of the nature of the fibrinogen component in fibrin sealants, the immuno-reaction of the non-reduced agarose SDS electrophoresis method was modified first established by Connaghan et al (1985) and by Proietti et al (1990). The modification of this semi-quantitative method initially designed to monitor fibrin crosslinked polymers and its degradation products in plasma, was used for monitoring the number of fibrinogen monomers/units and their amounts in each oligomer. In various fibrinogen preparations and in fibrin sealants.

Even though a detailed protocol for fibrinogen identification using agarose horizontal gel electrophoresis has been published in 1990, the method was unsatisfactory for the separation of high molecular wt. fibrinogen protein

(Proietti et al 1990). The method developed by Reines (1990) for the detection of vWF multimers to be used for the immunodetection of fibrinogen, was modified as follows: The Horizontal Electrophoresis was performed using the Reines et al (1990) detailed protocol for vWF multimers, however, the detection of fibrinogen multimers and the analysis method were of Proietti et al (1990), with some modifications (Proietti et al 1990). Briefly, samples were prepared by diluting to a protein concentration of 0.1mg/ml in a solution of 70 mM Tris, 4 mM EDTA, 2.4 % SDS, 9 M urea and 0.1 g/L bromophenol blue added as a marker and incubated at 60°C for 30 min. Agarose was dissolved to a final concentration of 1 % (stacking gel), 1.6 % (cathode gel) and 2 % (separating gel) by boiling in 70 mM Tris, 4 mM EDTA and 4 % SDS (stacking gel buffer) or 100 mM Tris, 150 mM glycine and 0.1 % SDS (cathode gel buffer and cathode buffer) or 200 mM Tris, 100 mM glycine and 0.4 % SDS (separating gel buffer). Gels (cathode, stacking and separating gels) were cast at >50°C and allowed to polymerize for at least 2 hours for each gel. Samples were loaded at 0.6-0.9 µl per well, then electrophoresis was performed toward the anode at 600 - 650 volts/running hours at a constant temperature of 16°C on a flat bed electrophoresis apparatus (Pharmacia Fine Chemicals, Sweden). The running buffer was connected to the gel via 4-8 layers of filter paper immersed in electrode buffer (the anode buffer had half the strength of the cathode buffer). Electrophoresis proceeded until the tracking dye had migrated a distance of 6-8 cm. After electrophoresis, the gel was removed, the stacking and cathode gels taken away and the separating gel washed in transfer buffer (25 mM Tris-HCl, 192 mM glycine pH 8.3 buffer, diluted 1:2 in 20 % methanol) for approximately 1 minute.

Immunoblotting was performed onto a Nitrocellulose membrane (Optiran BA-S83 S&S Germany), cut to size, using the capillary blotting procedure (Reines et al 1990). After protein transfer, the nitrocellulose membrane was carefully removed from the capillary blotting unit and the residual protein binding capacity blocked by immersion in blocking buffer (0.5% I-Block Tropix, USA. 0.05% Sodium Azide in PBS) for 1 hour. Immunodetection of fibrinogen

multimers was done using a goat polyclonal anti-human fibrinogen antibody (Sigma, USA), and a rabbit polyclonal anti-goat immunoglobulin alkaline phosphatase conjugate (Sigma, Mo USA) was used for immunostaining.

SDS-Polyacrylamide electrophoresis was done in a vertical electrophoretic unit (Hoefer Scientific Instruments, San Francisco, CA USA) using a reducing system containing 250 mM Tris, 40 % glycerol, 4% SDS and 4 % β -Mercaptoethanol (loading buffer). The separating gel was prepared as a gradient from 6 to 12 % acrylamide concentration (from a 30 % acrylamide and 0.8 % bisacrylamide stock solution) containing 10 % ammonium persulfate and 0.02 % TEMED. The samples were mixed with 4 x loading buffer to $\frac{1}{4}$ of the final volume and heated in a dry block at 95°C for 5 minutes. The gel was run at 200V and the electrophoresis was stopped when the Bromophenol Blue reached the bottom of the gel. Immunodetection was performed using a semi-dry immuno-blotter (Hoefer Scientific Instruments, San Francisco, CA USA) onto a nitrocellulose membrane using the same antibodies and protocol as for the horizontal electrophoresis.

Figure 1 shows the immunodetection of fibrinogen multimers of three plasma fractionations in-process sources of fibrinogen. One source derived from Paste I was supplemented with Factor FXIIIa. (From right to left) Lanes 1-6: cryoprecipitate; Lanes 7-12: purified fibrinogen; Lanes 13-18: purified Fibrinogen + FXIIIa; Lanes 19-21: solvent detergent paste I; Samples were taken during the process of the invention: Lanes 1, 7, 13 and 19: samples taken at the beginning of the process. Lanes 2, 8, 14 and 20: samples taken after the addition of sucrose and glycine. Lanes 3, 4, 9, 10, 15, 16 and 21: taken after incubation at 60°C for 17 hours. Lanes 5, 11, and 17: taken after dialysis and ultrafiltration. Lanes 6, 12, 18, and 22: taken after the formulation with 10% Tranexamic acid and 2% Arginine. All the samples were loaded at 0.7-0.9 μ g per lane.

Figure 2 shows the imunodetection of fibrinogen multimers electrophoresed on agarose SDS gel. Fibrinogen samples are of eight batches of the product of the present invention - lane 4: #C31102; lane 5: C30092; lane 6: C29081; lane 7: C27071; lane 8: C25061; lane 9: C22052; lane 10: 14032; lane 11: C12022. Four additional commercial sources of fibrinogen were added - lane 1: Tisseel™; lane 2: BeriPlast; lane 3: BioCol and lane 12: fibrinogen from Enzyme Research Inc. All the samples were loaded at 0.65µg per lane.

Figure 3, shows the immunodetection of fibrinogen multimers of three commercial preparations of fibrin sealants: From left to right. Lane 12: Tisseel™; lane 11: BioCol; lane 10: Fibrinogen from Enzyme research Inc.; lane 9: product prepared according to the method of the invention. Other lanes are fibrinogen production in process samples taken after the following steps: lane 1: cryoprecipitate resuspension sample-BC01; lane 2: aluminum alhydrogel absorption sample-BC02; lane 3: SD treatment -BC04; lane 4: SD removal-BC06; lane 5: after pasteurization-BC09; lane 6: addition of stabilizers - BC10; lane 7: final concentration-BC11, lane 8: sterile bulk-BC12. All the samples were loaded at 0.65µg per lane.

Figure 4 shows a western blot-immunodetection of various formulations of urea non-reduced fibrinogen multimers of product prepared according to the method of the invention, run by Agarose-SDS gel electrophoresis. Lane 1: no Arginine and no Tranexamic acid; lane 2: 2% Arginine; lane 3: 2% Arginine and 5% Tranexamic acid; lane 4: 2% Arginine and 10% Tranexamic acid; lane 5: 10% Tranexamic acid, lane 6: 1% Arginine and 10% Tranexamic acid; lane 7: 4% Arginine and 10% Tranexamic acid, lane 8: Fibrinogen from Enzyme Research Inc.; lane 9: Tisseel™; lane 10: BioCol. All samples were loaded at 0.65 µg protein per lane.

Figure 5 shows western blot-immunodetection of various formulations of reduced fibrinogen multimers of product prepared according to the method of the invention, migrated on PAGE-SDS gel electrophoresis. Lane 1: No Arginine

and no Tranexamic acid; lane 2: 2% Arginine; lane 3: 2% Arginine and 5% Tranexamic acid; lane 4: 2% Arginine and 10% Tranexamic acid; lane 5: 10% Tranexamic acid; lane 6: 1% Arginine and 10% Tranexamic acid; lane 7: 4% Arginine and 10% Tranexamic acid, lane 8: Fibrinogen from Enzyme Research Inc.; lane 9: Tisseel™. All samples were loaded at 10 µg protein per lane.

The initial fibrinogen multimeric state in various fibrinogen sources and the effect of a production process on the polymerization was identified. Three fibrinogen sources were used: 1) Re-suspension of cryoprecipitate (Sample A, Omrix Biopharmaceuticals LTD (Israel)); 2) purified fibrinogen, derived from Cohn Fraction I (Sample B, Enzyme Research, USA) or purified fibrinogen supplemented with FXIIIa (Sample C, Enzyme Research USA) and 3) solvent detergent treated fraction I (Sample D, from OctaPharma (Austria). They were treated with the process of the present invention. Pasteurization, dialysis and formulation with Tranexamic acid and Arginine and at each stage of the procedure, samples have been taken to test the state of polymerization (see Table 1).

Samples labeled A1, B1, C1 and D1 are re-suspended fibrinogen samples that have been directly taken for testing (lanes 1,7,13 and 19 in figure 1).

Samples labeled A2, B2, C2 and D2 are re-suspended fibrinogen samples mixed with 65% (w/w) sucrose and 0.2 M glycine, incubated at 37°C for less than 4 hours, heated to 60°C to allow for complete sucrose dissolution (lanes 2,8,14 and 20 in figure 1).

Samples labeled A3, B3, C3 and D3 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours and incubated at 60°C for 17 hours (lanes 3,9,15 and 21 in figure 1).

Samples labeled A4, B4 and C4 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours. Then,

incubation at 60°C for 17 hours was performed and dialyzed against 10 mM citrate buffer 120 mM NaCl, 120 mM glycine and 1mM CaCl₂ (lanes 4,10 and 16 in figure 1).

Samples labeled A5, B5, C5 and D5 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours, incubated at 60°C for 17 hours, and dialyzed against 10 mM citrate buffer 120 mM NaCl, 120 mM glycine and 1mM CaCl₂. The resulting solution was then formulated with 10% Tranexamic acid and 2% Arginine (lanes 5,11 and 17 in figure 1). For additional clarifications see Table 1.

Table 1: Experimental design of fibrinogen treatments, sources, addition of Factor XIIIa, incubation at high temperature, formulation during the pasteurization with sucrose and glycine and addition of final product stabilizers, Tranexamic acid and Arginine. The effect of all these parameters on the fibrinogen multimeric state was tested.

Lane #	No. of sample	Name of sample	Procedure - end point of treatment	Incubation temperature	Incubation time at the last point
1	A1	Cryoprecipitate	-	-	0
2	A2	Cryoprecipitate	Sucrose, glycine	37°C	4h
3	A3	Cryoprecipitate	pasteurization	60°C	17h
4	A3'				
5	A4	Cryoprecipitate	dialysis ultrafiltration	and RT	3h+2.5h
6	A5	Cryoprecipitate	add 10%TEA, Arginine	2% RT	1h
7	B1	Purified Fibrinogen		RT	0
8	B2	Purified Fibrinogen	Sucrose, glycine	37°C	2h
9	B3	Purified Fibrinogen	pasteurization	60°C	17h
10	B3'				
11	B4	Purified Fibrinogen	dialysis ultrafiltration	and RT	3h+2.5h
12	B5	Purified Fibrinogen	add 10%TEA, Arginine	2% RT	1h
13	C1	Purified Fibrinogen +FXIII		RT	0
14	C2	Purified Fibrinogen +FXIII	Sucrose, glycine	37°C	2h
15	C3	Purified Fibrinogen +FXIII	pasteurization	60°C	17h
16	C3'				
17	C4	Purified Fibrinogen +FXIII	dialysis ultrafiltration	and RT	3h+2.5h
18	C5	Purified Fibrinogen FXIII	add 10%TEA, Arginine	2% RT	1h
19	D1	Fraction I S/D		RT	0
20	D2	Fraction I S/D	Sucrose, glycine	37°C	1.5h
21	D3	Fraction I S/D	pasteurization	60°C	17h

Figure 1 shows that the cryo-precipitate fibrinogen source has more fibrinogen multimers than the other fibrinogen sources, as it can be seen in bands 1, 7, 13 and 19. On the other hand, the 60°C incubation increased the number and the amounts of fibrinogen units per multimer and the percentage of multimeric fibrinogen. However, this effect is most evident in purified fibrinogen and in Fraction I (lanes 9 and 15). The effect of FXIIIa addition is marginal, probably since all fibrinogen sources from plasma contain traces of FXIII.

The effect of fibrinogen source is further demonstrated in Figure 2. Figure 2 shows multimers of fibrinogen of fibrin sealant produced using the process of the invention, from cryoprecipitate after double virus inactivation (solvent detergent and pasteurization) and stabilizer addition from eight batches. Figure 2 also presents the Multimeric State of four fibrinogen concentrates from commercial sources (Purified fibrinogen Enzyme Research, TissuCol, Biocol and Beriplast). As can be noted from the figure, purified fibrinogen (Enzyme research Inc.) derived from Fraction I is primarily a monomer. However that preparation contains a small amount of fibrinogen dimeric structure. On the other hand, commercial fibrinogen products, which compose part of fibrin sealants, contain fibrinogen multimers up to pentamers while the fibrin sealant of the present invention has high multimeric fibrinogen structures reaching up to octamers or nonamers. Furthermore, the batch to batch reproducibility, relative to the high multimeric state of fibrinogen, is evident.

The first part of the study revealed that pasteurization has a major effect on the polymerization of fibrinogen from Fraction I. However, the effect on cryoprecipitate was not fully demonstrated. In the second part of the study, the changes in the Multimeric State of fibrinogen during the production process and the batch to batch variability were examined. Samples were withdrawn from the process and the material was subjected to examination by immunodetection after agarose gel electrophoresis. Figure 3 shows fibrinogen immunodetection of samples obtained following each production step: cryo-resuspension - lane 1; alhydrogel absorption lane 2; SD treatment - lane 3,

SD removal - lane 4, post-pasteurization - lane 5, stabilizer addition - lane 6, final concentration - lane 7, sterile bulk - lane 8 and final container-lane 9, purified fibrinogen-lane 10 and the two commercial fibrinogen concentrates (Tissel and Biocol)-lanes 11 and 12 respectively. The figure clearly shows that even though the cryo-resuspension contains six-mers (lane 1), the addition of stabilizers (lane 6) promoted fibrinogen polymer formation since lane 6, sample after stabilizers addition, contains at least one more multimeric unit than the previous sample, after pasteurization (lane 5). This clearly indicates that the addition of Arginine and Tranexamic acid had an effect on the fibrinogen multimeric state.

In order to examine the effect of each of the stabilizer, a sample was withdrawn from the process prior to stabilizer addition. At this point, several combinations of Arginine and Tranexamic acid were formulated containing between 0 - 4 % and 0 - 10 % (w/w) respectively. The different formulations were frozen, stored for seven days and thawed at 37°C. After thawing, the samples, together with purified fibrinogen and commercial fibrin concentrates, were run on a horizontal SDS-Agarose non reduced and vertical reduced β -mercaptoethanol SDS-PAGE) electrophoresis. The results are shown in figures 4 (horizontal SDS-Agarose non-reduced) and 5 (SDS-PAGE reduced β -mercaptoethanol SDS-PAGE). The horizontal agarose gel electrophoresis followed by specific immunodetection of fibrinogen shows that addition of Arginine promotes multimer formation but the main contribution to its effect is done by the addition of Tranexamic acid. This finding is supported by samples that contains 0% Arginine and 10 % TEA which produced more and larger fibrinogen multimers than 2 % Arginine and 0% TEA. The same phenomenon can be seen on the reduced β -mercaptoethanol SDS-PAGE conducted on the same samples (Figure 5). A new band with a molecular weight above 250 kD appears in all formulations but not in the formulation with no stabilizers and not in the commercial concentrated fibrinogen tested (fibrinogen from Enzyme Research Inc and TisseelTM). Furthermore, in the samples formulated with 10% Tranexamic acid, this high molecular weight band is much more intense than

other formulations, indicating an increase in the ratio of a high number of multimeric units. Another observation that can be made from the figure is the absence of almost all the γ - γ and α - γ fibrinogen dimers from samples produced according to the method of the invention that are quite abundant in other fibrinogen concentrates (Siebenlist and Mossesson, 1996). This suggests that the increase of fibrinogen polymers is at the expense of fibrinogen dimers. Moreover, the addition of Tranexamic acid and Arginine might stabilize the fibrinogen polymers or even drive the reaction towards a higher multimeric state.

It was established that native fibrinogen, left undisturbed, forms reversible temperature- dependent aggregates (Becker and Waugh, 1980; Becker, 1987). Based on light scattering and viscosity examinations Rozemfeld and Vasileva, (1991) suggested that fibrinogen undergoes spontaneous self-association through its D domain to form flexible end to end polymer chains. They found that self-association accrued more rapidly when factor XIIIa was present, but the spatial arrangement of the fibrinogen molecules remained the same. Masesson and Siebenlist 1995 supply direct ultra-structural evidence for such associations (Siebenlist and Mossesson, 1996). However, the extent of these polymers has never been investigated in highly concentrated fibrinogen of the commercial available fibrin sealants. Furthermore in this manuscript, for the first time, evidence is been given for a fractionation production process which might lead to such high multimeric appearance of fibrinogen.

As noted in figure 1 and 3, Cryoprecipitate production resulted in a high multimeric structure. However, during the production process, some of the highest molecular multimers (seven to nine fibrinogen units) are lost, probably due to filtration and precipitation. Later steps in the process of the invention, such as pasteurization and stabilization with negatively charged amino acid, recovered the high multimeric state and stabilized it even during a prolonged storage (see figure 2). Other commercial product such as BeriPlast™ and Tisseel™ also derived from cryoprecipitate, however, the final product contains

lower multimeric fibrinogen. Both contain 4-5 fibrinogen units whereas the product prepared according to the process of the invention contains about 8-9 fibrinogen units. A different manufacturing process and different formulation may explain this difference. Both are formulated with Aprotinin and a low concentration of amino acid whereas the fibrin sealant prepared according to the process of the invention is formulated with a high concentration of Tranexamic acid and Arginine. This high salt concentration probably facilitated the formation of the high molecular wt. multimers. BioCol on the other hand is produced from a modification of Cohn fraction I. This starting material has a low multimeric state (see figure 1), probably because of the presence of a high concentration of ethanol which suppresses the enzymatic activity of FXIII during the precipitation of fibrinogen.

The clinical relevance of the multimeric state has to be assessed carefully by conducting well designed animal experiments. Since such experiments involve strong commercial interest, these experiments have to be performed in a well-controlled manner. The animal model consists of spraying of adhesive/fibrin sealant to control bleeding caused by partial liver resection in heparinized rabbit. Results show that the bleeding time and the amount of glue used were significantly shorter and lower for the fibrin sealant prepared according to the process of the present invention than in other fibrinogen concentrate tested (BeriPlast™ and Tisseel™). These outcomes may indicate that it has better adhesive properties to tissue than other glues. There are also reports indicating that it seems to work also at low temperature in indications such as liver transplantation. One explanation of the high adherence to tissue may be related to the high multimeric state of it as compared to the other fibrin sealants tested. These large molecules, some of them of more than 2×10^6 D molecular wt, are structurally very similar to the fibrin network. These protein macromolecules have better adhesive forces than their monomeric units, very similar to the phenomenon known in vWF Multimers.

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Claims

1. Use of fibrinogen multimers having at least 6 fibrinogen units as an ingredient of a fibrin sealant.
2. Use according to claim 1 having 6 - 15 fibrinogen units as an ingredient of a fibrin sealant.
3. Use according to claim 1 wherein the fibrinogen multimers have 6 to 9 fibrinogen units.
4. Use according to any of claims 1 to 3 wherein the amount of fibrinogen multimers having 6 to 9 fibrinogen units is at least 5% (w/w) of total fibrinogen.
5. A method for the production of a fibrin sealant comprising fibrinogen multimers having at least 6 fibrinogen units comprising the steps of
 - resuspending cryoprecipitate
 - adding sucrose to yield a concentration of 60 to 70% (w/w),
 - adding glycine to yield a concentration of 0.1 to 0.3 M,
 - heating to 60°C for 15 - 20 hours,
 - removing the glycine and the sucrose by dialysis,
 - adding a protease inhibitor.
6. The method of claim 5, wherein the dialysis is conducted against a buffer comprising NaCl, glycine and CaCl₂.

7. The method of claim 5, wherein the protease inhibitor is tranexamic acid in a concentration of 8 - 12% (w/w) and/or arginine in a concentration of 1 - 3% (w/w).
8. A fibrin sealant comprising multimeric fibrinogen of at least 6 fibrinogen units.

Abstract

Use of fibrinogen multimers having at least 6 fibrinogen units as an ingredient for a fibrin sealant.

1 / 5

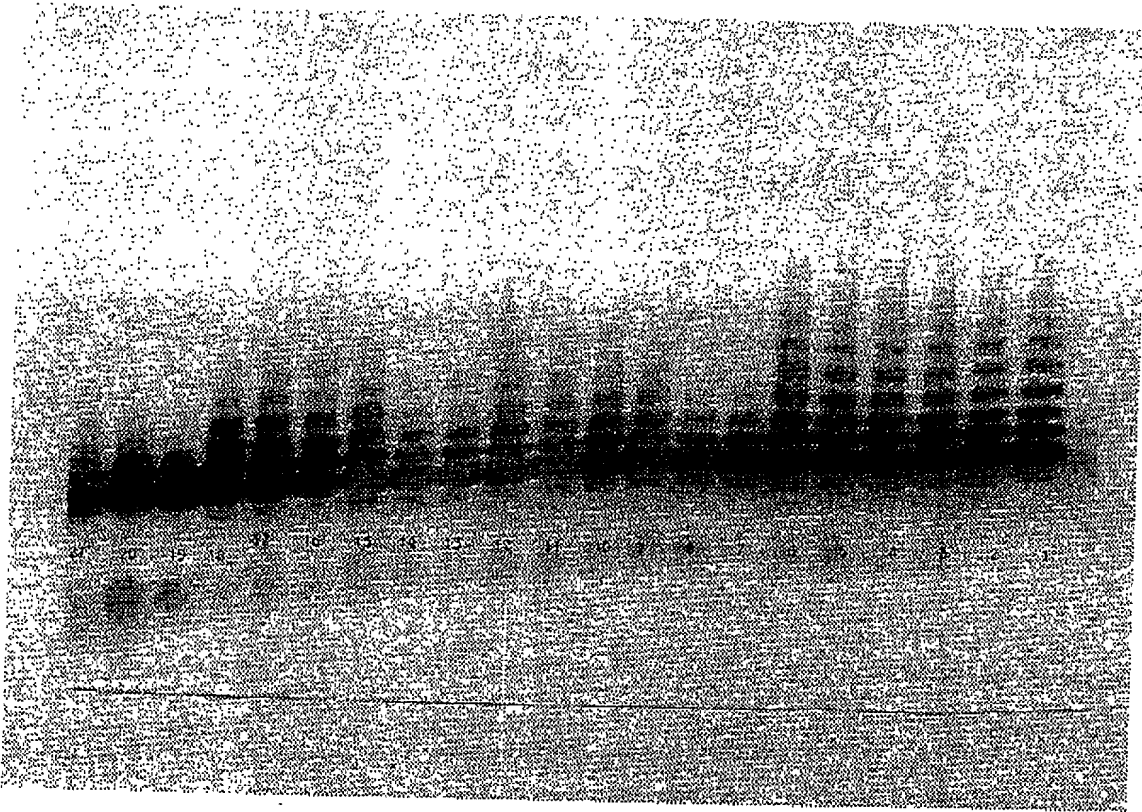
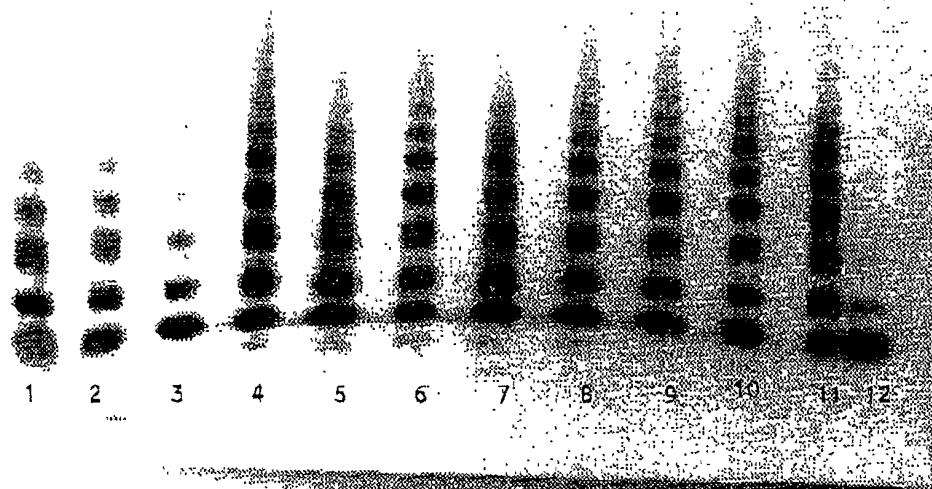


Figure No. 1 -

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**Figure No 2**

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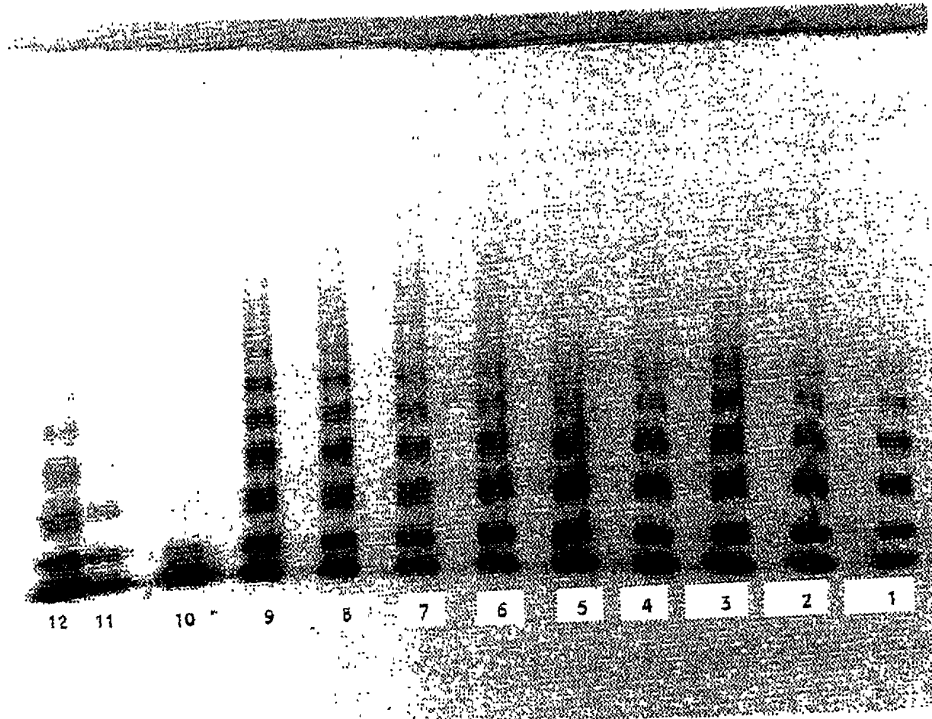


Figure No. 3:

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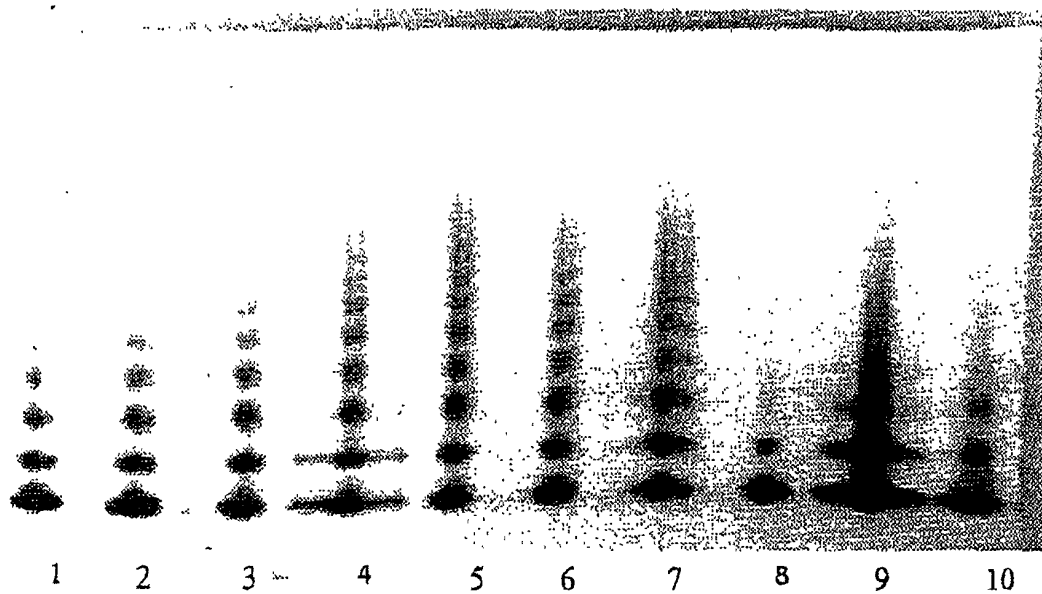


Figure No.4.

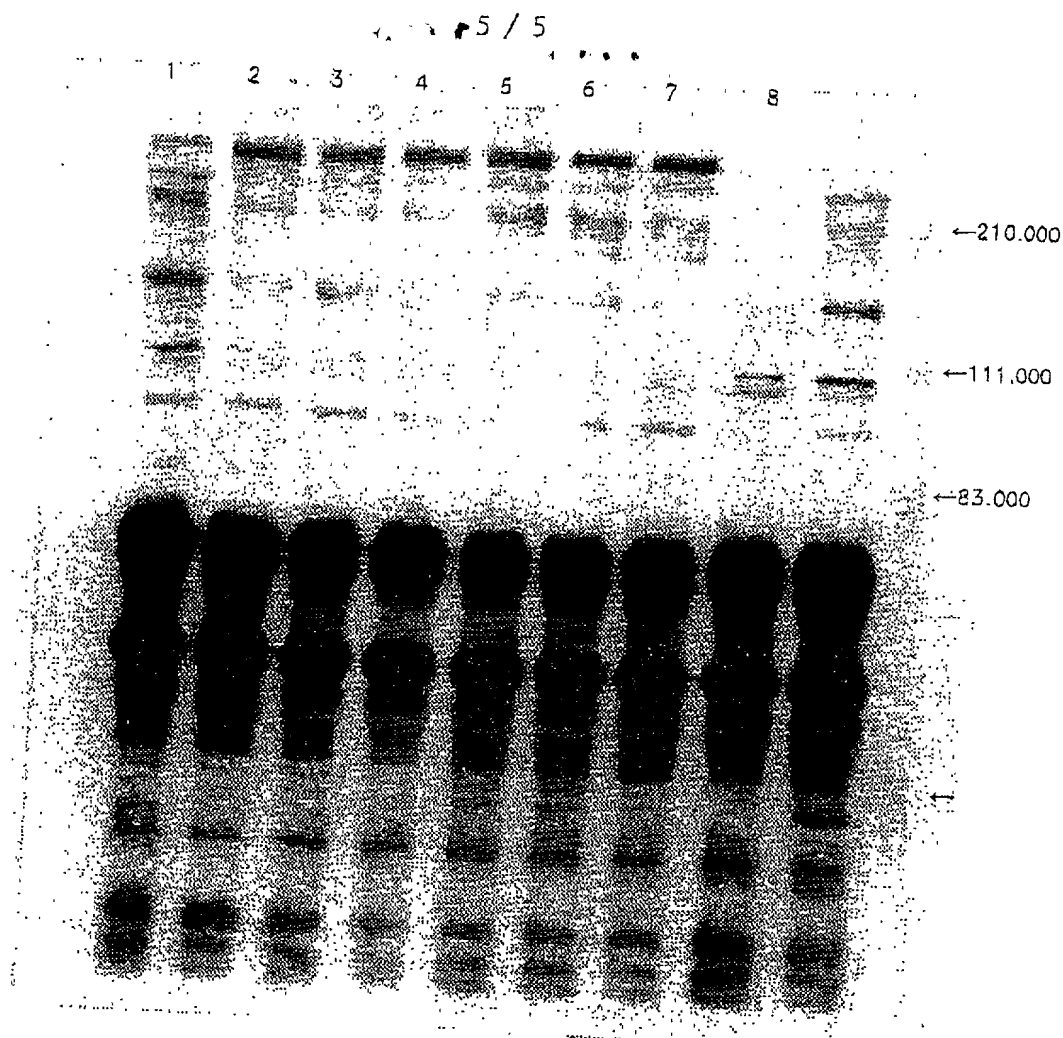


Figure 5

DECLARATION
AND POWER OF ATTORNEY
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ALL PATENTS, INCLUDING DESIGN
FOR APPLICATION BASED ON PCT; PARIS CONVENTION;
NON PRIORITY; OR PROVISIONAL APPLICATIONS

As a below named inventor, I declare that my residence, post office address and citizenship are stated below next to my name, the information given herein is true, that I believe that I am the original, first and sole inventor (if only one name is listed at 201 below), or an original, first and joint inventor (if plural inventors are named below at 201-203, or on additional sheets attached hereto) of the subject matter which is claimed and for which patent is sought on the invention entitled:

Use of fibrinogen multimers

which is described and claimed in:

☒ PCT International Application No. PCT/EP 00/07843

filed August 11, 2000

☐ the attached specification

☐ the specification in application Serial No. _____

filed _____

(if applicable) and amended on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

99 115 958.3

Europe

13/08/1999

(Number)

(Country)

(Day/Month/Year Filed)

Priority Claimed

☒ Yes

☐ No

00 108 499.5

Europe

19/04/2001

(Number)

(Country)

(Day/Month/Year Filed)

☒ Yes

☐ No

(Number)

(Country)

(Day/Month/Year Filed)

☐ Yes

☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Application No. _____

Filing Date _____

Application No. _____

Filing Date _____

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status: patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys (Registration No.) to prosecute this application, receive and act on instructions from my agent, and transact all business in the Patent and Trademark Office connected therewith. HARVEY B. JACOBSON, JR. (20,851); D. DOUGLAS PRICE (24,514); JOHN CLARKE HOLMAN (22,769); MARVIN R. STERN (20,640); ALLEN S. MELSER (27,215); MICHAEL R. SLOBASKY (26,421); JONATHAN L. SCHERER (29,851); IRWIN M. AISENBERG (19,007); WILLIAM E. PLAYER (31,409); YOON S. HAM (45,307) and NATHANIEL A. HUMPHRIES (22,772)

SEND CORRESPONDENCE TO: CUSTOMER NO. 00136

or

JACOBSON, PRICE, HOLMAN & STERN
PROFESSIONAL LIMITED LIABILITY COMPANY
400 SEVENTH STREET, N.W.
WASHINGTON, D.C. 20004

DIRECT TELEPHONE CALLS TO:

(please use Attorney's Docket No.) (202) 638-6666

JACOBSON, PRICE, HOLMAN & STERN
PROFESSIONAL LIMITED LIABILITY COMPANY

*Inventor(s) name must include at least one unabbreviated first or middle name.

201	FULL NAME * OF INVENTOR	FAMILY NAME <u>BAR</u>	GIVEN NAME <u>Lily</u>	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Rehovot</u>	STATE OR FOREIGN COUNTRY <u>Israel</u>	COUNTRY OF CITIZENSHIP <u>Israel</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>P.O. Box 619</u>	CITY <u>Rehovot</u>	STATE OR COUNTRY <u>Israel</u> ZIP CODE <u>76106</u>
202	FULL NAME * OF INVENTOR	FAMILY NAME <u>NUR</u>	GIVEN NAME <u>Israel</u>	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Rehovot</u>	STATE OR FOREIGN COUNTRY <u>Israel</u>	COUNTRY OF CITIZENSHIP <u>Israel</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>P.O. Box 619</u>	CITY <u>Rehovot</u>	STATE OR COUNTRY <u>Israel</u> ZIP CODE <u>76106</u>
203	FULL NAME * OF INVENTOR	FAMILY NAME	GIVEN NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201*	SIGNATURE OF INVENTOR 202*	SIGNATURE OF INVENTOR 203*
DATE <u>28.02.2001</u>	DATE <u>28.02.2001</u>	DATE

☐ Additional inventors are named on separately numbered sheets attached hereto.

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